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Stress: A Potential Role in Neurodegenerative Disease

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## **Table of Contents**

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Foreword.....</b>	<b>3</b>
<b>Introduction.....</b>	<b>5</b>
<b>Body.....</b>	<b>5-7</b>
<b>Key Research Accomplishments.....</b>	<b>7</b>
<b>Reportable Outcomes.....</b>	<b>7</b>
<b>Conclusions.....</b>	<b>7</b>
<b>References.....</b>	<b>8</b>
<b>Appendices.....</b>	<b>8</b>

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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## **5. INTRODUCTION**

Neurons within the central nervous system are highly dependent upon aerobic metabolism. Reduced oxygen (hypoxia/ischemia) can lead to neuronal dysfunction, and if the exposure is prolonged and severe it can cause neurodegeneration. The long-range goal of our research is to identify and characterize the signal transduction pathways that promote protection against hypoxia stress. We hypothesize that dysfunction of these pathways during hypoxia can lead to neuronal damage and death. The specific objective of the current project is to determine the role of the mitogen- and stress-activated signal transduction pathways in regulating the cellular response to hypoxia. Our studies are performed on a clonal cell line that was derived from pheochromocytoma tumors. Because these cells (PC12) display many characteristics that common to neurons, we use them as an *in vitro* model system for studying signal transduction and gene regulatory pathways that regulate the neuronal responses to environmental stresses such as hypoxia.

## **6. BODY**

During the past year (year 1) two major studies were performed which focused on Objectives 1 and 2 of the Statement of Work. The first study was undertaken to examine the role of the p38 kinase and mitogen-activated protein kinase (MAPK) signal transduction systems in mediating the response to hypoxia in PC12 cells. The second study was performed to determine the "down-stream" targets of these signaling systems that might be involved in mediating tolerance to hypoxia. A summary of the findings from this research is provided below. Detailed descriptions of this research are provided in the attached journal articles.

*Study 1.* We conducted an experimental study to determine the role of the p38 kinase pathway in regulating the cellular response to hypoxia. This pathway plays a critical role in responding to cellular stress and survival (Widmann et al., 1999; Su and Karin, 1996). We performed experiments in PC12 cells to determine if p38 kinase, a poorly characterized stress-related pathway, is activated by hypoxia. There are five separate isoforms of p38 kinase ( $\alpha$ ,  $\beta_1$ ,  $\beta_2$ ,  $\delta$ , and  $\gamma$ ). We performed experiments to determine if any of these isoforms are activated by hypoxia. We found that exposure to moderate hypoxia (5% O<sub>2</sub>) progressively stimulated phosphorylation and activation of the p38 $\gamma$  and p38 $\alpha$  only. We also found that prolonged hypoxia induced phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK). Interestingly, hypoxia also down regulated cyclin D1, a gene known to be regulated negatively by the p38 kinase pathway and involved in regulation of cell proliferation and differentiation (Lavoie et al., 1996). The activation of cyclin D1 by hypoxia was partially blocked by a pharmacological agent (SB203580) that inhibits the p38 kinase pathway. Studies were then undertaken to determine which isoform of p38 kinase is responsible for regulation of cyclin D1. We found that overexpression of a kinase-inactive form of p38 $\gamma$  was able to significantly inhibit the activation of cyclin D1 by hypoxia. This is potentially an important finding for understanding the basic mechanisms by which PC12 cells develop a tolerance to hypoxia. In an on-going study we have evidence that a cyclin A-like protein

is upregulated by hypoxia. One of the goals of our future research is to characterize this regulation. It is possible that cyclin D1 and other cyclins (cyclin A) are involved in regulation of the hypoxia tolerant phenotype. If so, activation of the p38 pathway and subsequent activation of cyclins might provide protection against the harmful effects of prolonged hypoxia on neurons.

**This study was published in the Journal of Biological Chemistry 274: 23570-23576, 1999. This article is attached.**

*Study 2:* We examined the role of the MAPK pathway on activation of EPAS1, a recently discovered hypoxia-activated transcription factor (Tian et al., 1997). We found that EPAS1 (which has 48% homology with HIF1 $\alpha$ , another hypoxia-activated transcription factor) is phosphorylated in PC12 cells during hypoxia, and that MAPK, but not p38 kinase, is involved in the activation of EPAS1. Pretreatment of PC12 cells with an inhibitor (PD98059) of MEK, an intermediate enzyme in the MAPK pathway, completely blocked hypoxia-activation (transactivation of a reporter gene that contained EPAS1 binding sites, i.e. HREs) of EPAS1. Interestingly, pharmacological blockade of MEK with PD98059 failed to prevent phosphorylation of EPAS1 during hypoxia in PC12 cells. This indicates that other kinases which are downstream from MAPK are involved in the hypoxia-induced activation of EPAS1. We also found that dominant-negative disruption of ras, the customary entry point into the MAPK pathway, did not prevent phosphorylation of MAPK or the trans-activation of the HRE-reporter gene. Thus, hypoxia activates MAPK in a ras independent manner. Moreover, pharmacological blockade of calmodulin blocked both the hypoxia-induced phosphorylation of MAPK and the EPAS1 trans-activation of the HRE reporter gene. Thus it appears that multiple signal transduction pathways including p38 kinase, MAPK and calcium/calmodulin are involved in the regulation of hypoxia-responsive genes in PC12 cells.

**This study was published in the Journal of Biological Chemistry 274: 33709-33713, 1999. This article is attached.**

In summary, during the first year of this grant we have been successful in showing that the p38 kinase and the mitogen-activated protein kinase pathways are activated by hypoxia and potentially play an important role in protecting cells against hypoxic stress. During the next year we will continue to pursue this line of investigation with special focus on understanding how these pathways regulate genes that confer a hypoxia tolerant phenotype. We hypothesize that hypoxia tolerant phenotypes are less likely to be damaged by hypoxia. Hypoxia associated damage in the central nervous system could be a major contributing factor to neurodegeneration.

**A short invited News and Views article written by the Principal Investigator (David E. Millhorn) describing the effect of hypoxia on signal transduction pathways and gene regulatory mechanisms is also attached.**

**A detailed description of protocols, methods and experimental approaches is provided in the attached journal articles. Statistical application and data analysis is also provided in the attached articles.**

## **7. KEY RESEARCH ACCOMPLISHMENTS:**

- First evidence that the p38 kinase signal transduction pathway is activated by hypoxia.
- Activation of the p38 kinase pathway is isoform specific; only the  $\alpha$  and  $\gamma$  isoforms of this enzyme are activated by hypoxia.
- Cyclin D1 is inhibited by hypoxia via the p38 kinase pathway.
- We were first to show that the mitogen-activated protein kinase (MAPK) is also activated by hypoxia in PC12 cells. (The MAPK system is a parallel system to the p38 kinase pathway).
- We were first to show that the hypoxia induced transcription factor EPAS1 is phosphorylated during hypoxia and that this phosphorylation leads to transactivation of genes that contain the HRE sequences.
- We also showed that the calcium/calmodulin pathway interacts with the MAPK pathway to phosphorylate and activate EPAS 1

## **8. REPORTABLE OUTCOMES:**

- Two full journal and one short review (News and Views) articles were published. These are provided in the appendix to this progress report.
- P.William Conrad received his Ph.D. degree in June 2000. His research during the 1999-2000 was supported in part by this grant. He is first author on the two attached research papers.

## **9. CONCLUSIONS:**

Significant progress was made on objectives 1 and 2 of the Statement of Work. The primary findings were that the p38 and MAPK signal transduction pathways play a primary role in regulating the response to hypoxia in PC12 cells. We further showed that these pathways activate downstream targets (cyclin D1, cyclin A-like, and EPAS1) that are potential mediators of hypoxia tolerance. We conclude that the p38 kinase and MAPK pathways play a primary role in conferring a hypoxia tolerant phenotype to PC12 cells, a model cell line for neurons.

**10. REFERENCES** (Journal of Biological Chemistry format)

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Su, B., and Karin, M. (1996) *Curr. Biol.* 8, 402-411.

Tian, H., McKnight, S., and Russell, D.W. (1997) *Genes Dev.* 11, 72-82.

Widmann, C., Gibson, S., Jarpe, B., and Johnson, G.I. (1999) *Physiol. Rev.* 79, 143-180.

**11. APPENDICES:**

(see attached journal articles)

## Selective Activation of p38 $\alpha$ and p38 $\gamma$ by Hypoxia

ROLE IN REGULATION OF CYCLIN D1 BY HYPOXIA IN PC12 CELLS\*

(Received for publication, January 26, 1999, and in revised form, May 15, 1999)

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Hypoxic/ischemic trauma is a primary factor in the pathology of a multitude of disease states. The effects of hypoxia on the stress- and mitogen-activated protein kinase signaling pathways were studied in PC12 cells. Exposure to moderate hypoxia (5% O<sub>2</sub>) progressively stimulated phosphorylation and activation of p38 $\gamma$  in particular, and also p38 $\alpha$ , two stress-activated protein kinases. In contrast, hypoxia had no effect on enzyme activity of p38 $\beta$ , p38 $\beta_2$ , p38 $\delta$ , or on c-Jun N-terminal kinase, another stress-activated protein kinase. Prolonged hypoxia also induced phosphorylation and activation of p42/p44 mitogen-activated protein kinase, although this activation was modest compared with nerve growth factor- and ultraviolet light-induced activation. Hypoxia also dramatically down-regulated immunoreactivity of cyclin D1, a gene that is known to be regulated negatively by p38 at the level of gene expression (Lavoie, J. N., L'Allemand, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) *J. Biol. Chem.* 271, 20608–20616). This effect was partially blocked by SB203580, an inhibitor of p38 $\alpha$  but not p38 $\gamma$ . Overexpression of a kinase-inactive form of p38 $\gamma$  was also able to reverse in part the effect of hypoxia on cyclin D1 levels, suggesting that p38 $\alpha$  and p38 $\gamma$  converge to regulate cyclin D1 during hypoxia. These studies demonstrate that an extremely typical physiological stress (hypoxia) causes selective activation of specific p38 signaling elements; and they also identify a downstream target of these pathways.

Mammalian cell function is critically dependent on a continuous supply of oxygen. Even brief periods of oxygen deprivation (hypoxia/ischemia) can result in profound cellular and tissue damage. Thus, it is vital that organisms meet changes in O<sub>2</sub> tension with appropriate cellular adaptations; however, the specific intracellular pathways by which this occurs are not well delineated. The stress- and mitogen-activated protein kinase (SAPK<sup>1</sup> and MAPK) pathways play a critical role in responding to cellular stress and promoting cell growth and sur-

vival (1, 2). We therefore investigated the effect of hypoxia on the SAPK and MAPK signaling pathways.

SAPKs and MAPKs are the downstream components of three-member protein kinase modules (3). Five homologous subfamilies of these kinases have been identified, and the three major families include p38/SAPK2/RK, JNK/SAPK, and p42/p44 MAPKs/ERKs (1–6). In general, the stress-activated protein kinases (p38 and JNK) are activated primarily by noxious environmental stimuli such as ultraviolet light, osmotic stress, inflammatory cytokines, and inhibition of protein synthesis (7–10). However, increasing evidence suggests that, at least under certain conditions, these pathways can also be activated by mitogenic and neurotrophic factors (11, 12). In contrast, p42/p44 MAPKs are stimulated primarily by mitogenic and differentiative factors in a Ras-dependent manner (5, 13, 14), although these enzymes can also be activated by certain environmental stressors (1–3). Thus, we hypothesized that hypoxia, a prevalent physiological stressor in many disease states, may regulate the activity of the SAPK and MAPK signaling pathways.

The pheochromocytoma cell line PC12 is a catecholaminergic, excitable cell type that has been used widely as an *in vitro* model for neural cells (15). Upon prolonged exposure to nerve growth factor (NGF), PC12 cells decrease proliferation and extend neurite-like processes (15). It has also been shown that PC12 cells are an O<sub>2</sub>-sensitive cell type that provides a useful system to study the effects of hypoxia on catecholaminergic gene expression (16–21). PC12 cells are exquisitely sensitive to hypoxia in that very small reductions in atmospheric O<sub>2</sub> (from 21 to 15% O<sub>2</sub>) dramatically induce tyrosine hydroxylase gene expression and mRNA stability (16, 17). Hypoxia also induces activation of the cAMP response element-binding protein (CREB) and *c-fos* transcription factors in this cell type (17, 20, 21). In addition, PC12 cells tolerate moderate hypoxia well in that they maintain greater than 95% cell viability for up to 72 h of exposure to hypoxia (5% O<sub>2</sub>, ~50 mm Hg) (22). Finally, PC12 cells also express hypoxia-regulated ion channels, as shown by the finding that PC12 cells depolarize during hypoxia via an oxygen-regulated K<sup>+</sup> current (23, 24) and secrete dopamine and norepinephrine (25, 26). Thus, this cell type is an ideal system in which to study regulation of intracellular signaling systems by hypoxia.

In the current studies, we have used this cell line to investigate the effect of hypoxia on the SAPK and MAPK signaling pathways. We show that hypoxia selectively activates the p38 $\gamma$  and p38 $\alpha$  isoforms of the p38 pathway in this cell type. The p38 $\gamma$  subtype in particular is most strongly targeted by hypoxia. Furthermore, we identify cyclin D1, a gene that has been

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$\S$  The abbreviations used are: SAPK, stress activated protein kinase; MAPK, mitogen activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NGF, nerve growth

factor; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropane-sulfonic acid; PDZ, PSD-95, Discs-Large, ZO-1; RK, reactivating kinase; MAPKAP, mitogen-activated protein kinase activated protein.

shown previously to be regulated by p38 (27), as a downstream target of hypoxia-induced activation of both p38 and p38 $\gamma$ .

## EXPERIMENTAL PROCEDURES

**Reagents and Antibodies**—SB203580 and NGF were obtained from Calbiochem. Anisomycin, sorbitol, and anti-FLAG M2 antibody were obtained from Sigma. Anti-p38 (C-20), anti-JNK1 (C-17), and anti-ERK2 (C-14) antibodies, protein G-coupled agarose for immunoprecipitations, and anti-cyclin D1 (C-20) antibodies for Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A-coupled Sepharose was obtained from Amersham Pharmacia Biotech. MAPKAP kinase-2 assay kits and myelin basic protein were from Upstate Biotechnology, Inc. (Lake Placid, NY), and c-Jun (1-79) was from Santa Cruz Biotechnology. Phospho- and total p38 and phospho- and total p42/p44 MAPK antibodies were obtained from New England Biolabs (Beverly, MA). All cell culture media and reagents were obtained from Life Technologies, Inc.

**Cell Culture**—PC12 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 20 mM HEPES (pH 7.4), 10% fetal bovine serum, and with penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). Prior to experimentation, cells were grown to approximately 85% confluence in 35- or 60-mm tissue culture dishes (Corning) in an environment of 21% O<sub>2</sub>, 5% CO<sub>2</sub>, balanced with N<sub>2</sub>. Hypoxia was achieved by exposing cells to 5% O<sub>2</sub>, 5% CO<sub>2</sub>, balanced with N<sub>2</sub> for various times in an O<sub>2</sub>-regulated incubator (Forma Scientific, Marietta, OH). In previous studies, we have shown that the partial pressure of O<sub>2</sub> in the media of cells exposed to 5% O<sub>2</sub> is in the range of 50–55 mm Hg (16).

Stable PC12 cell lines were created by transfecting cells with either FLAG-tagged p38 $\gamma$ AF in pcDNA3 (28) or the empty pcDNA3 vector, using Trans-Fast reagent (Promega, Madison, WI), at a charge ratio of 1:1, according to the manufacturer's recommended conditions. Individual clones expressing the kinase-inactive form of p38 $\gamma$  (p38 $\gamma$ AF) were selected in the presence of G418 (0.4 mg/ml). Clones were screened for p38 $\gamma$ AF expression by immunoblotting whole cell lysates with an anti-FLAG antibody, as described below.

**Western Blotting**—After exposure to hypoxia, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by adding 0.2 ml/35-mm dish of a lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.2 mM sodium vanadate, 10 mM sodium fluoride, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 2  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin. Lysates were sonicated for 1 s with a microultrasonic cell disrupter (Kontes, Vineland, NJ) and then centrifuged for 10 min at 14,000  $\times$  g at 4 °C to remove the Triton-insoluble fraction. The protein concentration was determined by the method of Bradford (Bio-Rad), and gel samples were prepared by adding sample buffer containing final concentrations of 50 mM Tris (pH 6.7), 2% SDS, 2%  $\beta$ -mercaptoethanol, and bromphenol blue as a marker. Gel samples were boiled for 2 min, and then 20–100  $\mu$ g of protein was loaded on 7.5% or 9% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) using standard electroblotting procedures. Nitrocellulose membranes were blocked with 5% nonfat dry milk or 5% bovine serum albumin, for phosphotyrosine immunoblots. Blocking solutions were prepared in a buffer containing 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.1% Tween 20 (PBST).

Blots were immunolabeled overnight at 4 °C with antibodies recognizing the dual phosphorylation motif at Thr<sup>180</sup> and Tyr<sup>182</sup> of p38 (1:500) or with an antibody that equally recognizes phospho- and dephospho-p38 (1:3,000). The phosphorylation state of p42/p44 MAPK was evaluated using an antibody that specifically recognizes phospho-Tyr<sup>204</sup> MAPK (1:1,000) or an antibody that equally recognizes phospho- and dephospho-MAPK (1:1,000). Cyclin D1 expression was analyzed using an anti-cyclin D1 antibody (1:2,500). FLAG-tagged p38 protein kinases were detected with an anti-FLAG M2 monoclonal antibody (1:500). Immunoblots were washed in several changes of PBST at room temperature and then incubated with anti-rabbit Ig linked to horseradish peroxidase or, for FLAG and cyclin D1, an anti-mouse Ig linked to horseradish peroxidase (Amersham Pharmacia Biotech). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's recommended conditions and quantified using densitometric analysis with an ImagePro digital analysis system (Media Cybernetics, Silver Springs, MD). Immunoreactivity for all proteins evaluated was linear over at least a 3-fold range of protein concentrations.

**Immune Complex Kinase Assays**—For MAPK and SAPK assays, cells were grown to 70% confluence on 35-mm tissue culture dishes. For p38 kinase assays, cells on 35-mm dishes were transiently transfected with

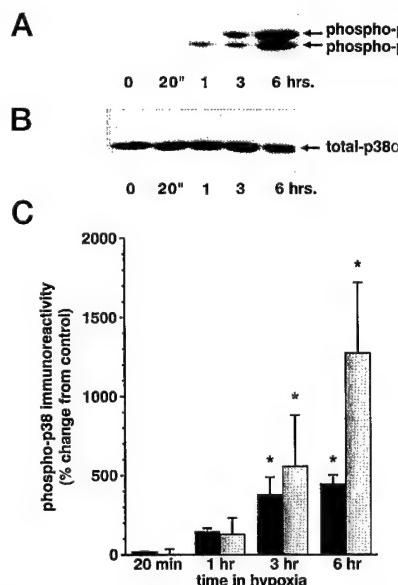
5  $\mu$ g of FLAG-p38, FLAG-p38 $\beta$ , FLAG-p38 $\beta$ 2, FLAG-p38 $\gamma$ , FLAG-p38 $\delta$ , or pcDNA3, using Trans-Fast reagent at a charge ratio of 1:1, according to the manufacturer's recommended conditions. These constructs have been described previously (5, 28–30). Cells were then exposed to normoxia, hypoxia, or UV light (300 J/m<sup>2</sup>), or sorbitol (300 mM) 48 h after transfection. Cells were then washed with ice-cold PBS and harvested by adding 0.3 ml of buffer A (50 mM Tris (pH 7.4), 2 mM EDTA, 2 mM EGTA, 0.5 mM sodium vanadate, 10 mM  $\beta$ -glycerophosphate, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml aprotinin, and 0.1% (v/v)  $\beta$ -mercaptoethanol). Cell lysates were centrifuged for 10 min at 14,000  $\times$  g at 4 °C to pellet the Triton-insoluble fraction. FLAG-tagged p38 isoforms were immunoprecipitated from 200  $\mu$ g of total cellular protein using 10  $\mu$ g of anti-FLAG M2 monoclonal antibody coupled to agarose and followed by rocking at 4 °C for 2–24 h. Immunoprecipitation of MAPK or JNK was achieved by adding 0.5  $\mu$ g of ERK2 or 1  $\mu$ g of JNK antibody to lysates containing 500  $\mu$ g of total cellular protein and rocking at 4 °C for 2–4 h. 50  $\mu$ l of a 10% (w/v) suspension of protein A-Sepharose beads was then added, and the reaction slurry was allowed to rock at 4 °C for 2–24 h. The immunoprecipitation complex was washed twice with 0.5 ml of ice-cold fresh buffer A, twice with PBS, and twice with kinase assay buffer (containing 20 mM MOPS (pH 7.2), 25 mM  $\beta$ -glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol). In addition to buffer A described above, the kinase assay reaction mixture contained final concentrations of 7.5 mM MgCl<sub>2</sub>, 50  $\mu$ M ATP containing 20  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and either 10  $\mu$ g of myelin basic protein for p38 and p42/p44 MAPK assays, or 10  $\mu$ g of c-Jun (1-79) for JNK assay, in a final volume of 100  $\mu$ l. Reactions were initiated by the addition of 10  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]ATP (NEN Life Science Products) and incubated for 20 min shaking at 30 °C. Reactions were stopped by the addition of Laemmli SDS sample buffer containing  $\beta$ -mercaptoethanol and bromphenol blue. Samples were boiled for 2 min and run on either 15% SDS-polyacrylamide gels for analysis of p38 and p42/p44 MAPK or 9% SDS-polyacrylamide gels for JNK enzyme activity. Kinase activity was measured as the amount of <sup>32</sup>P incorporation into the specific substrate proteins as quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). MAPKAP kinase-2 assays were performed essentially as described previously (20) except that cell lysates were rapidly frozen in a dry ice/ethanol bath to facilitate cell lysis. Lysates were then thawed and processed for MAPKAP kinase-2 activity using an immunoprecipitation kinase kit (Upstate Biotechnology Inc.) according to the manufacturer's recommended conditions.

**Flow Cytometry**—Flow cytometry was performed as described previously (31). PC12 cells were grown to approximately 70% confluence on 35-mm tissue culture dishes. After normoxic or hypoxic treatment for 24 h, cells were harvested by adding 150  $\mu$ l of 0.05% trypsin. 1 ml of a solution containing 10% fetal bovine serum in PBS was added to quench the trypsin. Cells were then centrifuged and resuspended in 100  $\mu$ l of a freezing buffer containing 250 mM sucrose, 5% dimethyl sulfoxide, and 40 mM sodium citrate. Cells were stored at –80 °C until preparation for flow cytometry. 50  $\mu$ l from each cell sample was aliquoted and then lysed by the addition of 100  $\mu$ l of a solution containing 0.5% Nonidet P-40 and 0.5 mM EDTA in PBS. 1  $\mu$ l of RNase (10 mg/ml, Qiagen, Santa Clarita, CA) was also added, and the cell mixture was then rocked for 15 min at room temperature. The samples from each tube were added to 1 ml of a solution containing 50  $\mu$ g/ml propidium iodide in PBS. Samples were analyzed on a Coulter Epics XL (Beckman-Coulter Co., Miami, FL) and analyzed using a WinCycle software package (Phoenix Flow Systems, San Diego).

## RESULTS

Hypoxia is an extremely common physiological stressor. To investigate the effects of hypoxia on the stress- and mitogen-activated signaling pathways, PC12 cells were exposed to 5% O<sub>2</sub> for various times, between 20 min and 6 h. Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then immunoblotted with an antibody specific for Thr<sup>180</sup>/Tyr<sup>182</sup>-phosphorylated p38 $\alpha$ . Phosphorylation at these sites is both necessary and sufficient for enzymatic activation of p38 $\alpha$  (5). It can be seen in Fig. 1A that exposure to hypoxia progressively induced phospho-p38 immunoreactivity in two closely migrating bands. Phospho-p38 blots were then stripped and reblotted with an antibody that equally recognizes phospho- and dephospho-p38 $\alpha$  (i.e. total p38 $\alpha$ ). Fig. 1B shows that the lower phospho-p38 immunoreactive protein shown in Fig.

## Activation of Specific Isoforms of p38 by Hypoxia

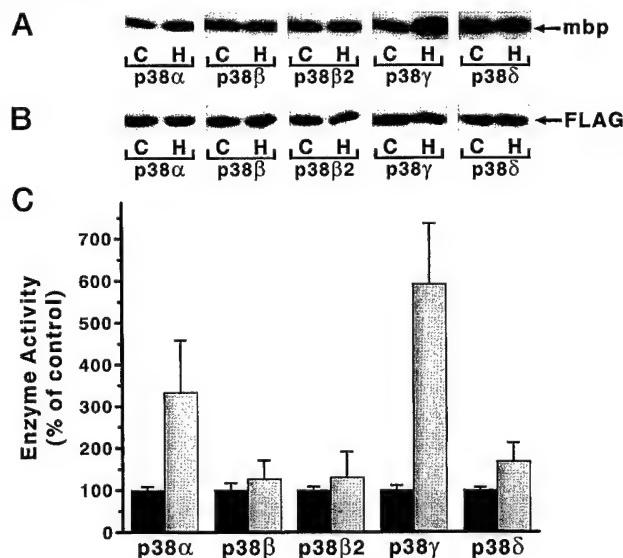


**FIG. 1. Effect of hypoxia on p38 $\alpha$  and p38 $\gamma$  phosphorylation state.** PC12 cells were exposed to hypoxia (5% O<sub>2</sub>) for various times between 0 and 6 h, as indicated. *Panel A*, representative immunoblot illustrating the effect of hypoxia on phospho-p38 $\alpha$  and phospho-p38 $\gamma$  immunoreactivity. *Panel B*, the blot shown in *panel A* was stripped and reprobed with an antibody that equally recognizes phospho- and dephospho-p38. *Panel C*, immunoreactivity levels of phospho-p38 $\alpha$  (black bars) and phospho-p38 $\gamma$  (shaded bars) are expressed as average percent change from control  $\pm$  S.E. and represent six dishes in each group, performed in two separate experiments. Phospho-p38 immunoreactivity was quantified by densitometry (\* $p < 0.01$ , by  $\chi^2$  test).

1A corresponded to p38 $\alpha$ , as determined by alignment of films using luminescent markers affixed directly to the blot. As shown in Fig. 1B, hypoxia did not alter the total amount of p38 $\alpha$  protein. Of the time points examined, maximal hypoxia-induced phosphorylation of p38 $\alpha$  occurred at 6 h, where there was an average 4.5-fold increase in p38 $\alpha$  phosphoimmunoreactivity (Fig. 1C). The upper phospho-p38 immunoreactive band was identified as the p38 $\gamma$  isoform, as described below. Phosphoimmunoreactivity of p38 $\gamma$  was increased more strongly by hypoxia, with an average of 12.7-fold increase over control levels by a 6-h exposure to hypoxia (Fig. 1C). These results suggest that both p38 $\alpha$  and p38 $\gamma$  are activated by hypoxia. Phosphorylation of p38 $\alpha$  and p38 $\gamma$  declined somewhat but was still elevated above control levels up to 24-h exposure to hypoxia (data not shown).

The upper phospho-p38 immunoreactive band shown in Fig. 1A was identified as p38 $\gamma$  by stripping and reblotting with a specific antibody raised against full-length recombinant p38 $\gamma$  (28), an isoform of p38 also known as ERK6 and SAPK3 (32, 33). Alignment of the resulting films showed that p38 $\gamma$  comigrated exactly with the upper phospho-p38 immunoreactive protein (data not shown). Although p38 $\beta$  and p38 $\delta$  were also expressed in PC12 cells, neither of these proteins comigrated with p38 $\gamma$ , as determined using specific antibodies for the p38 $\beta$  and p38 $\delta$  subtypes (data not shown).

To characterize further the effects of hypoxia on p38 enzyme activity, PC12 cells were transfected with FLAG epitope-tagged versions of p38 $\alpha$ , p38 $\beta$ , p38 $\beta_2$ , p38 $\gamma$ , or p38 $\delta$ . Cells were then exposed to either normoxia (21% O<sub>2</sub>) or hypoxia (5% O<sub>2</sub>, 6 h). The various kinases were then immunoprecipitated with an anti-FLAG antibody, and immune complex kinase assays were performed, as described under "Experimental Procedures." As shown in Fig. 2A, hypoxia stimulated both p38 $\alpha$  and p38 $\gamma$  enzyme activity. In contrast to these results, hypoxia did not significantly alter p38 $\beta$ , p38 $\beta_2$ , or p38 $\delta$  enzyme activity.



**FIG. 2. Effect of hypoxia on enzyme activity of the various p38 isoforms.** PC12 cells were transfected with FLAG-p38 $\alpha$ , FLAG-p38 $\beta$ , FLAG-p38 $\beta_2$ , FLAG-p38 $\gamma$ , FLAG-p38 $\delta$ , or the pCDNA3 vector. After 48 h, cells were exposed to either control conditions (C, 21% O<sub>2</sub>) or hypoxia (H, 5% O<sub>2</sub>, 6 h). *Panel A*, enzyme activity of various p38 isoforms was determined in immune complex kinase assays by the amount of <sup>32</sup>P incorporation into myelin basic protein (mbp) as described under "Experimental Procedures." *Panel B*, whole cell lysates were immunoblotted for FLAG as described under "Experimental Procedures." *Panel C*, protein kinase activity of the various p38 isoforms after exposure to normoxia (black bars) or hypoxia (shaded bars) is expressed as average percent of control  $\pm$  S.E. and represents six to nine dishes in each group, performed in at least two separate experiments.

Hypoxia-induced changes in enzyme activity were not the result of differences in transfection efficiency as cell lysates blotted with anti-FLAG show equal amounts of the transfected protein (Fig. 2B). It can be seen that the effect of hypoxia on the p38 $\gamma$  isoform is by far the most robust (average 5.9-fold activation, Fig. 2C).

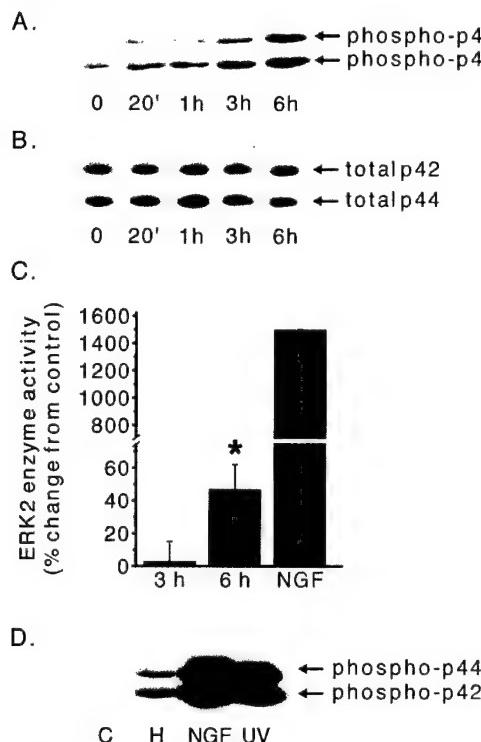
We next evaluated the effect of hypoxia on JNK, another SAPK. PC12 cells were exposed to hypoxia for various times, from 20 min to 6 h, and JNK enzyme activity was measured in an immune complex kinase assay, as described under "Experimental Procedures." Unlike its effects on p38, hypoxia did not alter JNK enzyme activity significantly, whereas exposure of cells to UV light increased JNK activity markedly (Fig. 3).

To determine the effect of hypoxia on p42/p44 MAPK, PC12 cells were again exposed to either normoxia (21% O<sub>2</sub>) or hypoxia (5% O<sub>2</sub>) for various times, between 20 min and 6 h. Samples of whole cell lysates were immunoblotted with either an antibody specific for tyrosine-phosphorylated (activated) p42/p44 MAPK or an antibody that equally recognizes phospho- and dephospho-p42/p44 MAPK (total MAPK). Hypoxia had no significant effect on the levels of phospho-p42/p44 MAPK at the earliest time points studied. However, exposure to hypoxia for 6 h caused an increase in the tyrosine phosphorylation of p42/p44 MAPK (Fig. 4, A and C). The total amount of p42/p44 MAPK was not affected by hypoxia, as shown in Fig. 4B. MAPK enzyme activity was measured directly by immune complex kinase assay. Fig. 4C shows that p42 MAPK enzyme activity, like the MAPK phosphorylation state, increased after 6 h of hypoxia. To compare the effects of hypoxia with the prototypical activators of MAPK, we also evaluated p42/p44 MAPK phosphorylation in response to NGF and UV light. In contrast to the rather modest effect of hypoxia, these stimuli caused a robust phosphorylation of p42/p44 MAPK (Fig. 4D).

The downstream transcription factors and protein kinases



**Fig. 3. Lack of effect of hypoxia on JNK activity.** PC12 cells were exposed to either hypoxia (5% O<sub>2</sub>) for various times between 0 and 6 h, as indicated, or to 300 J/m<sup>2</sup> UV light for 30 min. JNK was immunoprecipitated by the addition of 1 μg of anti-JNK1 polyclonal antibody as described under "Experimental Procedures." JNK enzyme activity was determined in an immune complex kinase assay by the amount of <sup>32</sup>P incorporation into c-Jun as quantified by PhosphorImager. Similar results were found in three separate experiments representing three dishes in each group.



**Fig. 4. Hypoxia modestly activates p42/p44 MAPK.** PC12 cells were exposed to hypoxia (5% O<sub>2</sub>) for various times between 0 and 6 h, as indicated. In panels A and B, lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies specific for either Tyr<sup>204</sup>-phosphorylated p42/p44 MAPK or total (phospho- and dephospho-) MAPK, as described under "Experimental Procedures." Panel A, representative immunoblot showing phospho-p42/p44 MAPK immunoreactivity at the various time points studied. Panel B, representative immunoblot showing total MAPK at the various time points studied. Results similar to those shown in panels A and B were observed in three separate experiments. Panel C, MAPK enzyme activity was determined in an immune complex kinase assay by the amount of <sup>32</sup>P incorporation into myelin basic protein as quantified by PhosphorImager. Data shown are representative of those obtained in two separate experiments and represent six dishes in each group. Panel D, representative immunoblot of Tyr<sup>204</sup>-phosphorylated p42/p44 MAPK immunoreactivity in lysates of PC12 cells exposed to normoxia (C, 21% O<sub>2</sub>), hypoxia (H, 5% O<sub>2</sub>), NGF (50 ng/ml), or 300 J/m<sup>2</sup> UV light (30 min). Similar results were found in two separate experiments representing six dishes in each group.

that are targeted by the p38 family are beginning to be elucidated (1–3, 34–43); however, very little is known about the specific genes that are regulated in response to activation of the p38 pathways. The cyclin D1 gene is one known target of p38, as Lavoie *et al.* (27) have shown that cyclin D1 gene expression is regulated negatively by p38 in CCl<sub>3</sub>9 cells. We therefore tested whether hypoxia regulated cyclin D1 levels in PC12 cells. We found that exposure to hypoxia (0, 3, 6, or 24 h at 5% O<sub>2</sub>) progressively down-regulated cyclin D1 levels, with an 81% decrease of cyclin D1 from control levels observed at 24 h (Table I). Pretreatment of cells with SB203580, a relatively selective inhibitor of p38 (43, 44), was able to reverse in part the down-

TABLE I  
Effect of hypoxia on cyclin D1 immunoreactivity in PC12 cells

Cells were exposed to hypoxia (5% O<sub>2</sub>) for various times between 0 and 24 h, as indicated. Cyclin D1 immunoreactivity was quantitated by densitometry.

Time in hypoxia (5% O <sub>2</sub> )	Cyclin D1 immunoreactivity <sup>a</sup>	
	h	%
0		100 (5)
3		*45.9 ± 3.4 (5)
6		*29.2 ± 3.0 (5)
24		*18.8 ± 2.0 (8)

<sup>a</sup> Values are the average percent of control ± S.E. (n). \* indicates *p* < 0.05 by  $\chi^2$  test.

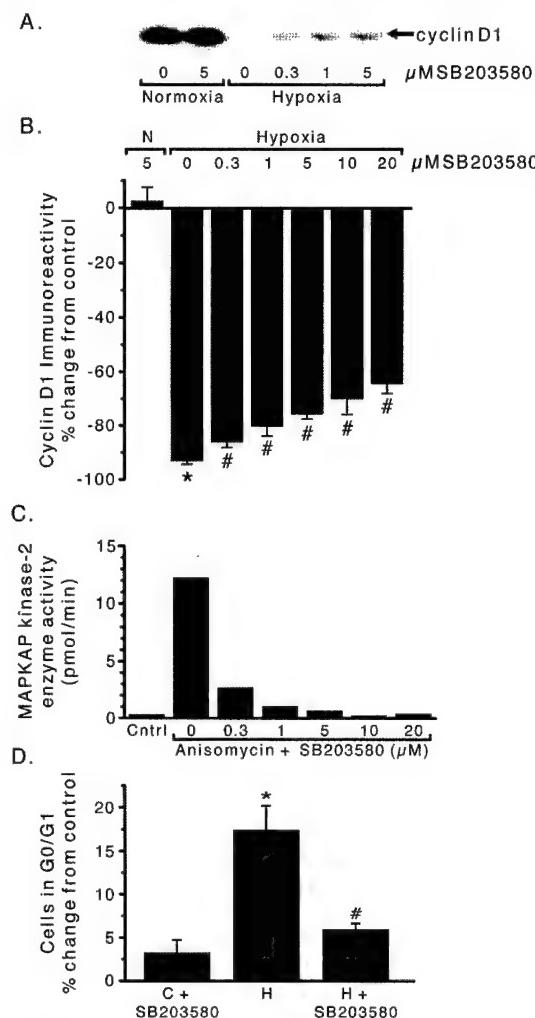
regulation of cyclin D1 by hypoxia in a dose-dependent manner (Fig. 5A). These results are expressed quantitatively in Fig. 5B, where it can be seen that pretreatment with SB203580 resulted in a partial, but statistically significant, recovery of cyclin D1 toward control levels. The inhibitory effect of SB203580 on hypoxic regulation of cyclin D1 was observed at low doses (0.3–1 μM) as was its inhibitory effect on anisomycin-activated MAPKAP kinase-2. MAPKAP kinase-2 is a protein kinase that is specifically phosphorylated and activated by the p38 family of protein kinases (Fig. 5C). The fact that SB203580 only partially reversed the effects of hypoxia may be because this drug does not inhibit the p38γ isoform (45–47), as discussed further below.

Cyclin D1 is a G<sub>1</sub> cyclin whose synthesis and associated cyclin-dependent kinase activity are generally required for progression through the G<sub>1</sub> phase of the cell cycle (48, 49). Our finding that hypoxia induces a down-regulation of cyclin D1 suggested that hypoxia may cause cells to accumulate in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. We therefore evaluated the relative percentage of cells in the various phases of the cell cycle in PC12 cells that were exposed to either normoxia or hypoxia for 24 h. Cells were stained with propidium iodide and analyzed by flow cytometry. It can be seen in Fig. 5D that hypoxia caused a 17.4% increase in the number of cells in G<sub>0</sub>/G<sub>1</sub>. Furthermore, pretreatment with SB203580 followed by a 24-h exposure to hypoxia was able to reverse in part this accumulation in G<sub>0</sub>/G<sub>1</sub>.

Our results show that hypoxia activates both p38α and p38γ; however, the p38γ isoform is insensitive to inhibition by SB203580 (45–47). This raised the possibility that p38γ might also contribute to the inhibition of cyclin D1 by hypoxia (*i.e.* the portion of the effect that was not inhibited by SB203580). To test this hypothesis, we generated stably transfected PC12 cell lines that express p38γAF, a kinase-inactive mutant of p38γ. Overexpression of a similar mutant (Y185F) has been shown previously to inhibit endogenous p38γ enzyme activity effectively (32). Fig. 6 shows that, compared with vector-transfected cells, the hypoxia-induced decrease in cyclin D1 is partially reversed in the p38γAF cell line. These results were confirmed in two separate clones and show that p38γ, like p38α, is involved in the down-regulation of cyclin D1 during hypoxia; however, pretreatment of p38γAF-expressing cells with SB203580 did not result in a further impairment of the effect of hypoxia on cyclin D1 expression (data not shown).

## DISCUSSION

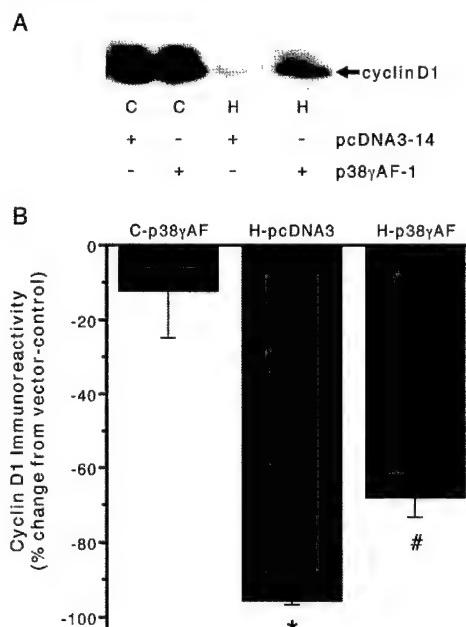
The signaling pathways involved in cellular responses and adaptations to hypoxia are very poorly understood. The PC12 cell line is a neural-like cell line that has been shown to respond to very small reductions in O<sub>2</sub> levels with changes in ion conductances (23, 24), protein phosphorylation (20, 22), and gene expression (16–21). These studies were aimed at identifying specific intracellular signaling pathways that are regulated by hypoxia in this cell type. We have shown that moder-



**FIG. 5. Hypoxia inhibits cyclin D1 and causes accumulation at G<sub>0</sub>/G<sub>1</sub> via a partially p38-dependent mechanism.** PC12 cells were exposed to either normoxia (C, 21% O<sub>2</sub>) or hypoxia (H, 5% O<sub>2</sub>) for 24 h in the presence or absence of increasing amounts of SB203580, as indicated. **Panel A**, representative immunoblot showing the effects of hypoxia and p38 inhibition on cyclin D1 immunoreactivity. **Panel B**, immunoreactivity levels of cyclin D1 are expressed as the average percent change from control ± S.E. and represent 6–12 dishes in each group performed in at least two separate experiments. \* indicates significant difference from control, and # indicates significant difference from hypoxia plus vehicle,  $p < 0.05$ , by  $\chi^2$  test. **Panel C**, cells were exposed to either vehicle (Cntr) or anisomycin (10  $\mu$ g/ml) for 20 min, in the presence of various levels of SB203580, as indicated. MAPKAP kinase-2 enzyme activity was measured in immune complex kinase assays, as described. **Panel D**, cells were pretreated with vehicle or 20  $\mu$ M SB203580 and then exposed to normoxia or hypoxia for 24 h. Cells were stained with propidium iodide and analyzed by flow cytometry as described under “Experimental Procedures.” Data are expressed as the percent change from control ± S.E. and represent seven dishes in each group, performed in two separate experiments. \* indicates significant difference from control, and # indicates significant difference from hypoxia plus vehicle,  $p < 0.05$ , by  $\chi^2$  test.

ate hypoxia (5% O<sub>2</sub>) selectively activates p38 $\gamma$  and p38 $\alpha$ , but not other isoforms of the p38 family of SAPKs. Furthermore, activation of both p38 $\gamma$  and p38 $\alpha$  is involved in the down-regulation of cyclin D1 during hypoxia. In contrast, another major SAPK, JNK, was not affected by hypoxia.

The p38 family of protein kinases consists of several isoforms, including p38 $\alpha$ , p38 $\beta$ , p38 $\beta$ 2, p38 $\gamma$ /SAPK3/ERK6, and p38 $\delta$ /SAPK4 (4, 10, 28–30, 32, 33, 45, 50, 51). These kinases are activated by a variety of stressors, including osmotic stress, UV light, inhibition of protein synthesis, and inflammatory cytokines; however, the mechanism by which these diverse



**FIG. 6. Role of p38 $\gamma$  in the hypoxia-induced decrease in cyclin D1.** PC12 cells stably transfected with a kinase-inactive form of p38 $\gamma$  or the empty expression vector pcDNA3 were exposed to hypoxia for 24 h, as indicated. **Panel A**, representative immunoblot showing the effect of p38 $\gamma$  inhibition on the hypoxia-induced decrease in cyclin D1. **Panel B**, immunoreactivity levels of cyclin D1 are expressed as average percent change from control ± S.E. and represent six dishes in each group performed in two separate experiments. \* indicates significant difference from control-pcDNA3,  $p < 0.05$ , by  $\chi^2$  test, and # indicates significant difference from hypoxia-pcDNA3,  $p < 0.05$ , by  $\chi^2$  test.

stimuli activate p38 kinases is not known. Our results demonstrate, for the first time, that physiological levels of hypoxia selectively activate p38 $\gamma$  and p38 $\alpha$ . Phosphorylation of p38 has been shown to occur after ischemia in heart and kidney (52). Taken together with our findings, it is possible that the hypoxic component of ischemia, rather than the other types of substrate depletion (glucose, ATP, etc.), results in the activation of p38 $\alpha$  and p38 $\gamma$ .

The p38 $\gamma$  isoform was most strongly targeted by hypoxia in PC12 cells. The molecular basis of this selectivity is not known, and in general, previous studies have found the closely related isoforms to be activated coordinately by various stressors (29, 46, 50, 51). Recent evidence suggests, however, that there may be unique physiological roles for p38 $\gamma$ . It has been shown that the carboxyl-terminal sequence -KEXTL of p38 $\gamma$  interacts with the PDZ domain of  $\alpha$ -syntrophin, a substrate that is phosphorylated by p38 $\gamma$  (53). Interestingly, p38 $\gamma$  is the only member of the currently known MAPK families to have a carboxyl-terminal PDZ domain binding sequence and is likely to interact with other PDZ domain-containing proteins. Many proteins with PDZ domains are localized to specific subcellular locations, such as synapses (54, 55). p38 $\gamma$  is enriched in skeletal muscle (28, 32, 51) but is also expressed at moderate levels throughout the central nervous system (51). Our results showing that hypoxia preferentially activates p38 $\gamma$  in a neural-like cell line suggests possible specialized roles for this enzyme in excitable cells.

The other major stress-activated signaling pathway acts through the JNK family of protein kinases (1–3). Like p38, the JNK family is activated by a number of stressors but is distinctive in its ability to phosphorylate the transcription factor c-Jun (6, 8). It has been reported previously that ischemia/reperfusion in the kidney and hypoxia/reoxygenation in cardiac myocytes induce activation of JNK (52, 56). These groups found

JNK activity to be activated by the reoxygenation event but not during the initial hypoxia or ischemia. It has also been reported recently that severe hypoxia ( $pO_2 \leq 0.01\%$ ) transiently activated JNK in human squamous carcinoma cells (57). In contrast, we found that neither hypoxia nor hypoxia plus reoxygenation (data not shown) between 20 min and 6 h stimulated JNK enzyme activity in PC12 cells. Clearly, various stressors can have different effects, depending on the specific cell type and its environment. The differential effects of hypoxia on p38 and JNK contribute to a small but growing number of stimuli that selectively activate p38 but not JNK (58).

Hypoxia (6 h, 5% O<sub>2</sub>) also caused a modest activation of p42/p44 MAPK in PC12 cells. It has been reported previously that HeLa cells respond to severe hypoxia with a rapid (within 15 min) but transient activation of p42/p44 MAPK (59). In PC12 cells, hypoxia induced a relatively small and delayed activation of p42/p44 MAPK compared with the robust and rapid activation induced by NGF or UV exposure.

It is of considerable interest to determine which downstream genes are regulated by p38 $\alpha$  and p38 $\gamma$  in response to hypoxia. A number of downstream kinases, including MAPKAP kinase-2/3 (34, 35), MAPK signal-integrating kinase (MNK) (36), and p38-regulated/activated protein kinase (PRAK) (37), as well as transcription factors and ternary complex factors, including C/EBP-homologous protein (CHOP), switch-activating protein (Sap1), myocyte-enhancer factor 2A (MEF2A), and MEF2C have been shown to be phosphorylated and activated by the p38 family of protein kinases (38–42); however, the specific genes that are regulated in response to activation of p38 and these transcription factors remain largely unknown. One gene that has been shown to be regulated by p38 is cyclin D1 (27). Activation of p38 strongly inhibits cyclin D1 gene expression in CCL39 cells (27). Likewise, hypoxia down-regulates cyclin D1 expression in PC12 cells. We showed further that p38 $\alpha$  is involved in this hypoxia-induced decrease in cyclin D1 levels, as the effect is partially blocked by low doses of SB203580, a relatively selective inhibitor of p38 (43, 44). The failure of SB203580 to reverse this effect completely may be because of activation of p38 $\gamma$ , which is insensitive to inhibition by SB203580 (45–47). p38 $\gamma$  is also involved in the regulation of cyclin D1, as overexpression of a kinase-inactive mutant (p38 $\gamma$ AF) partially reverses the decrease in cyclin D1 during hypoxia. However, pretreatment of PC12 cells overexpressing p38 $\gamma$ AF with SB203580 did not result in a further reversal of the effects of hypoxia on cyclin D1 expression (data not shown). It is not clear why SB203580 would be ineffective in this cell line, but it is possible that p38 $\gamma$ AF expression could impair both p38 $\alpha$  and p38 $\gamma$  function. Because both p38 $\alpha$  and p38 $\gamma$  have been shown to have identical upstream activators (46), p38 $\gamma$ AF may sequester activated MAP kinase kinase-3 (MKK3) and/or MKK6, thereby impairing the activity of any of its downstream p38 kinases. Alternatively, the stably transfected p38 $\gamma$ AF cells, because they are cultured in the presence of the selection drug (G418) may differ from the parental cell line in a number of ways that are difficult to assess.

Cyclin D1 has been implicated in regulating progression through the G<sub>1</sub> phase of the cell cycle (48, 49). The hypoxia-induced inhibition of cyclin D1 correlates with an increased accumulation of cells in G<sub>0</sub>/G<sub>1</sub> after exposure to hypoxia. This accumulation was also shown to be partially blocked by cotreatment of cells with SB203580. It is important to note that although there is a relative increase in the accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase, we did not observe a corresponding decrease in cell cycle progression during hypoxia. In fact, preliminary findings suggest that hypoxia may induce prolifera-

tion as measured by [<sup>3</sup>H]thymidine incorporation,<sup>2</sup> as has been reported in other cell lines (60–62). Such seemingly contradictory findings (a concomitant decrease in cyclin D1 levels with cellular proliferation) are not entirely incompatible. For example, cyclin D1 has been shown to be critical for growth factor-mediated proliferation (63). The role of cyclin D1 in hypoxia-induced proliferation, which likely proceeds via a different mechanism, is not known. In addition, hypoxia does not decrease the immunoreactivity of other major cyclins, including the S phase cyclin, cyclin A (data not shown), as would be predicted during inhibition of cell cycle progression. Furthermore, it has been shown that NGF induces cyclin D1 expression in PC12 cells (64). This increase in cyclin D1 is associated with a G<sub>1</sub> phase block and a decrease in proliferation, as PC12 cells begin to differentiate (65). Finally, cyclin D1 is now known to have other functions, separate from regulation of cyclin-dependent kinases. For example, cyclin D1 can associate with histone acetyltransferase, p300/CBP-associated protein (P/CAF) and facilitate estrogen receptor function (66). Thus, cyclin D1 levels do not always correlate with cell cycle progression, especially in this cell type. Clearly, further studies are required to elucidate the mechanism of hypoxia-induced regulation of cell cycle progression in PC12 cells.

Taken together, these studies demonstrate that hypoxia, an extremely typical physiological stress, causes specific regulation of the SAPK and MAPK signaling pathways. We also show that one isoform of p38, p38 $\gamma$ , is particularly strongly activated by hypoxia. This is, to our knowledge, one of the first demonstrations of selective activation of a particular subtype of a p38 family protein kinase. Furthermore, cyclin D1 levels are regulated by hypoxia via both p38 $\alpha$  and p38 $\gamma$ . Future studies are aimed at delineating the specific mechanisms by which a reduction in O<sub>2</sub> levels causes regulation of these pathways.

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# EPAS1 *trans*-Activation during Hypoxia Requires p42/p44 MAPK\*

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Hypoxia is a common environmental stress that regulates gene expression and cell function. A number of hypoxia-regulated transcription factors have been identified and have been shown to play critical roles in mediating cellular responses to hypoxia. One of these is the endothelial PAS-domain protein 1 (EPAS1/HIF2- $\alpha$ /HLF/HRF). This protein is 48% homologous to hypoxia-inducible factor 1- $\alpha$  (HIF1- $\alpha$ ). To date, virtually nothing is known about the signaling pathways that lead to either EPAS1 or HIF1- $\alpha$  activation. Here we show that EPAS1 is phosphorylated when PC12 cells are exposed to hypoxia and that p42/p44 MAPK is a critical mediator of EPAS1 activation. Pretreatment of PC12 cells with the MEK inhibitor, PD98059, completely blocked hypoxia-induced *trans*-activation of a hypoxia response element (HRE) reporter gene by transfected EPAS1. Likewise, expression of a constitutively active MEK1 mimicked the effects of hypoxia on HRE reporter gene expression. However, pretreatment with PD98059 had no effect on EPAS1 phosphorylation during hypoxia, suggesting that MAPK targets other proteins that are critical for the *trans*-activation of EPAS1. We further show that hypoxia-induced *trans*-activation of EPAS1 is independent of Ras. Finally, pretreatment with calmodulin antagonists nearly completely blocked both the hypoxia-induced phosphorylation of MAPK and the EPAS1 *trans*-activation of HRE-Luc. These results demonstrate that the MAPK pathway is a critical mediator of EPAS1 activation and that activation of MAPK and EPAS1 occurs through a calmodulin-sensitive pathway and not through the GTPase, Ras. These results are the first to identify a specific signaling pathway involved in EPAS1 activation.

Regulation of gene expression is a primary response by which cells adapt to changes in the environment. The mechanisms involved in regulation of gene expression in response to hypoxia are beginning to be understood. Transcription factors that are activated by hypoxia include the hypoxia-inducible factor (HIF1- $\alpha$ ),<sup>1</sup> c-fos, and CREB (1–4). HIF1- $\alpha$  has been

shown to be critical for hypoxia-induced regulation of a number of genes, including glycolytic enzymes, vascular endothelial growth factor, and erythropoietin (5–7). Recently, endothelial PAS-domain protein 1 (EPAS1, also known as HIF2- $\alpha$ , HLF, and HRF) was identified as a hypoxia-inducible transcription factor (8–10). EPAS1 is a basic helix-loop-helix transcription factor, which shares 48% sequence identity with HIF1- $\alpha$  (8). EPAS1 protein levels, like HIF1- $\alpha$  levels, are relatively low under basal conditions and accumulate upon exposure of cells to hypoxia (11). These factors then translocate to the nucleus and *trans*-activate target genes containing the sequence 5'-GCCCTACGTGCTGCTCA-3', which is commonly referred to as the hypoxia response element (HRE) (8, 12).

EPAS1 is expressed in many tissues and is particularly abundant in the type I oxygen-sensing cells of the carotid body (13). Type I cells act as the primary O<sub>2</sub> sensors in mammals and are responsible for matching changes in arterial pO<sub>2</sub> with appropriate changes in respiration (14). Our laboratory has used PC12 cells as a model system to study the biophysical and molecular properties of oxygen-sensing cells (15). There are a number of phenotypic similarities between type I and PC12 cells, including the presence of O<sub>2</sub>-sensitive K<sup>+</sup> channels, which are inhibited by hypoxia (16, 17). In addition, both PC12 cells and type I cells respond to hypoxia with an increase in tyrosine hydroxylase gene expression (18, 19). Finally, both cell types depolarize and secrete the neurotransmitter dopamine in response to hypoxia (20–22). We have therefore utilized PC12 cells to study the regulation of EPAS1.

The specific signaling pathways that are involved in HIF1- $\alpha$  and EPAS1 activation are almost completely unknown. In our previous studies, we measured the effects of hypoxia on the mitogen and stress-activated protein kinase pathways (MAPKs and SAPKs) (23). We found that moderate hypoxia (5% O<sub>2</sub>) activates p42/p44 MAPK, two closely related protein kinases that can lead to the phosphorylation and activation of a number of transcription factors (24). We therefore hypothesized that the MAPK pathway may be important for EPAS1 activation during hypoxia. Results from the current study show that the MAPK pathway is critical for EPAS1 activation, as the specific MEK1 inhibitor, PD98059, prevents EPAS1 *trans*-activation of the HRE. Interestingly, PD98059 had no effect on EPAS1 protein levels, suggesting that the MAPK pathway is involved in the activation of EPAS1, rather than the accumulation of EPAS1. We also show, for the first time, that EPAS1 itself is phosphorylated during hypoxia. However, EPAS1 is not directly phosphorylated by MAPK, suggesting that MAPK mediates its effects indirectly, possibly by recruiting other proteins critical for EPAS1 *trans*-activation. Finally, we show that MAPK-activation of EPAS1 during hypoxia occurs via a calmodulin-sensitive pathway and not through a Ras-dependent mechanism.

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<sup>1</sup> The abbreviations used are: HIF1- $\alpha$ , hypoxia-inducible factor; CREB, cyclic-AMP response element-binding protein; EPAS1, endothelial PAS-domain protein; HLF, HIF-like factor; HRF, HIF-related factor; HRE, hypoxia response element; PC12, pheochromocytoma; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein ki-

nase; DMEM, Dulbecco's modified Eagle's medium; NGF, nerve growth factor; CMZ, calmidazolium chloride; CBP, CREB-binding protein; VHL, von Hippel Lindau; PAGE, polyacrylamide gel electrophoresis.

## EXPERIMENTAL PROCEDURES

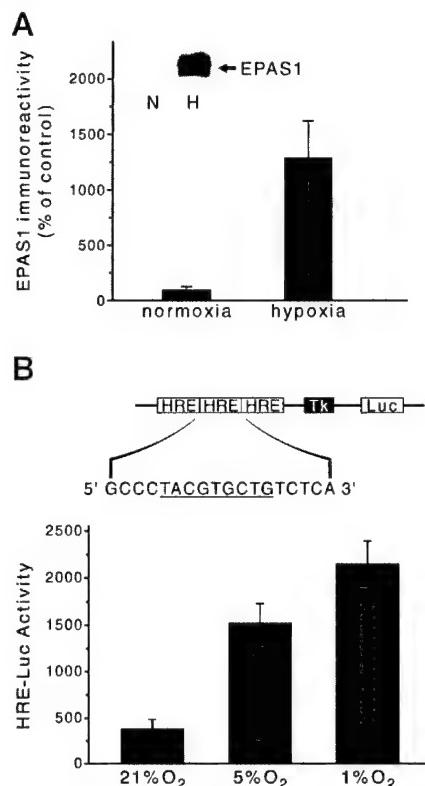
**Cell Culture and Materials**—PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Life Technologies, Inc.) supplemented with 20 mM HEPES, pH 7.4, 10% fetal bovine serum (Life Technologies, Inc.), and with penicillin (100 units/ml) and streptomycin (100 µg/ml). Prior to experimentation, cells were grown to approximately 85% confluence in 35- or 60-mm tissue culture dishes (Corning), or in 24-well plates for luciferase assays, in an environment of 21% O<sub>2</sub>, 5% CO<sub>2</sub>, balanced with N<sub>2</sub>. Hypoxia was achieved by exposing cells to various levels (10, 5, and 1%) of O<sub>2</sub>, 5% CO<sub>2</sub>, balanced with N<sub>2</sub> for various times in an O<sub>2</sub>-regulated incubator (Forma Scientific, Marietta, OH).

PD98059 was obtained from New England Biolabs (Beverly, MA). EPAS1 polyclonal antibody, the HRE-Luc reporter gene, and the EPAS1 cDNA were generous gifts from Dr. Steven L. McKnight (University of Texas Southwestern, Dallas, TX). pFC-MEK1 was obtained from Stratagene (La Jolla, CA). Additional EPAS1 polyclonal antibody was obtained from Novus Biologicals (Littleton, CO), and similar results were obtained with both antibodies. RasN-17 was a gift from Dr. J. Silvio Gutkind (National Institutes of Health, NIDR, Bethesda, MD). A c-fos-luciferase fusion reporter gene (fos-Luc) was constructed from a c-fos-β-galactosidase fusion gene construct, kindly provided by Dr. Tom Curran (St. Jude's Childrens Research Hospital, Memphis, TN). The β-galactosidase coding region was excised from the fos-lacZ plasmid (26) with NcoI and BamHI and replaced with the luciferase coding region from the pGL3-basic plasmid (Promega, Madison, WI). W13 was obtained from RBI (Natick, MA). Calmidazolum chloride was obtained from Calbiochem.

**Reporter Gene Assays**—PC12 cells were transfected with the hypoxia response element-luciferase (HRE-Luc) reporter gene using the Transfast transfection reagent according to the manufacturers recommended conditions (Promega). This reporter gene has been described previously (8, 12). PC12 cells seeded in 24-well plates at 60% confluence were transfected in triplicate with 3 µl of Transfast and 250 ng of HRE-Luc per well. In some experiments, 25–100 ng of EPAS1, pFC-MEK1, or RasN-17 was cotransfected with the HRE-Luc. In each transfection, pcDNA3 vector DNA was added to bring the total amount of DNA to 1 µg of DNA/well. Cells were switched to serum-free medium for 18 h prior to the start of the experiment. The following day (48 h post-transfection), PC12 cells were exposed to normoxia or hypoxia (1% O<sub>2</sub>) for 6 h. In other experiments, the effect of NGF on a c-fos reporter gene was evaluated. In these experiments, cells were cotransfected with 250 ng of the c-fos-luciferase reporter gene and varying amounts of an N-17 Ras expression plasmid in 24-well plates. After 48 h, cells were incubated with nerve growth factor (50 ng/ml, Alomone Labs, Jerusalem, Israel) for 6 h. To perform luciferase assays, cells were washed with phosphate-buffered saline and lysed in 200 µl of cell culture lysis reagent (Promega). Cell extracts were sonicated for 1 s with a microultrasonic cell disrupter (Kontes, Vineland, NJ). Twenty µl of cell extracts were then aliquoted into luminometer tubes (Promega). Fifty µl of luciferin substrate (Promega) was added to each tube and samples were analyzed in a luminometer (Turner Designs). We found previously that hypoxia inhibits expression of cytomegalovirus-β-galactosidase, Rous sarcoma virus-β-galactosidase, and SV40-β-galactosidase reporter genes.<sup>2</sup> Therefore, as in previous studies, luciferase activity was normalized to micrograms of protein per well (3). Protein samples varied by less than 15% between samples.

**Western Blotting**—Western blotting was performed as described previously (3, 23). For phospho-MAPK blots, membranes were immunolabeled with antibodies recognizing phospho-Tyr<sup>204</sup> MAP kinase (1:1000, New England Biolabs). EPAS1 protein expression was assayed using a rabbit polyclonal antibody directed against amino acids 1–10 of the EPAS1 protein at a dilution of 1:1000.

**Phosphorylation and Immunoprecipitation**—Experiments were performed essentially as described by Jewell-Motz et al. (27). Briefly, PC12 cells plated onto 100-mm dishes were washed twice with phosphate-free DMEM and then incubated at 37 °C in phosphate-free DMEM (Life Technologies, Inc.) for 30 min. Phosphate-free medium (5 ml/dish) containing 1 mCi/ml of [<sup>32</sup>P]orthophosphate and either Me<sub>2</sub>SO or PD98059 (50 µM) was added to the cells. After preincubation for 1.5 h, cells were exposed to normoxia or hypoxia (1% O<sub>2</sub>, 6 h). Cells were harvested by washing with ice-cold phosphate-buffered saline and scraping in 1 ml of a lysis buffer containing 25 mM Tris, pH 7.4, 1% Triton X-100, 0.5 mM sodium vanadate, 25 mM β-glycerophosphate, 1 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 2 µg/ml aprotinin. Whole cell lysates were precleared with 5 µl of rabbit IgG (Sigma) and 50 µl of a 10% (w/v) suspension of protein A-Sepharose beads. EPAS1 was immunoprecipitated using 10 µg of an EPAS1 poly-



**FIG. 1. EPAS1 protein accumulates and is activated by hypoxia.** PC12 cells were exposed to normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>, 6 h) followed by SDS-PAGE and immunoblotting with an α-EPAS1 antibody. *A*, immunoblot showing the effect of hypoxia on EPAS1 immunoreactivity. Results are representative of  $n = 6$  performed in two separate experiments. *B*, PC12 cells were seeded in 24-well dishes and transfected with the HRE-Luc reporter gene (250 ng/dish). 48-h post-transfection, cells were exposed to normoxia, or increasing levels of hypoxia, as indicated, and then assayed for luciferase activity as described under "Experimental Procedures." Data are representative of results performed in three experiments.

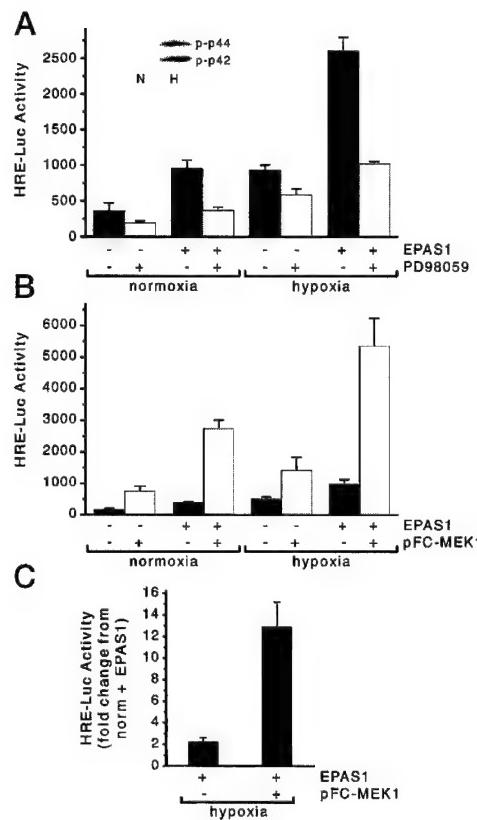
clonal antibody (Novus Biologicals) followed by the addition of 50 µl of a 10% (w/v) suspension of protein A-Sepharose beads. The reaction slurry was allowed to rock at 4 °C for 2 h. Immunoprecipitates were washed three times with lysis buffer and then subjected to 7.5% PAGE analysis. The gel was dried and analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

## RESULTS

As a first step toward characterizing the regulation of EPAS1 in PC12 cells, we evaluated EPAS1 protein levels following exposure to hypoxia. Fig. 1*A* shows that exposure to hypoxia (1% O<sub>2</sub>) for 6 h resulted in a 12-fold increase in EPAS1 protein levels. It has been established previously that EPAS1 can trans-activate an HRE-Luc reporter gene (8). We found that titrating the level of hypoxia from 21% O<sub>2</sub> to 1% O<sub>2</sub> resulted in a dose-dependent increase in HRE-luciferase activity (Fig. 1*B*).

We have shown recently that hypoxia specifically regulates certain members of the SAPK and MAPK family (23). We reported that moderate hypoxia (5% O<sub>2</sub>) induced a modest phosphorylation of MAPK. Fig. 2*A* shows results obtained when PC12 cells were exposed to more severe hypoxia (1% O<sub>2</sub>), which caused a pronounced phosphorylation of p42/p44 MAPK. Because the MAPK pathway is known to regulate a number of transcription factors, including c-fos, jun-B, CREB, and Elk-1 (28–30), we hypothesized that the MAPK pathway might be important for EPAS1 activation during hypoxia. To test this hypothesis, PC12 cells were cotransfected with the HRE-Luc reporter gene and a plasmid encoding the human EPAS1 cDNA or the empty expression vector, pcDNA3. Cells were then pretreated with either PD98059 (50 µM) or vehicle and exposed to

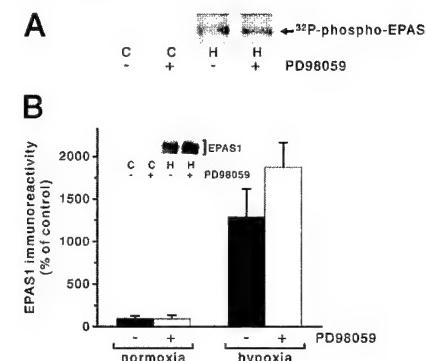
<sup>2</sup> D. Beittner-Johnson and D. E. Millhorn, unpublished observations.



**FIG. 2. p42/p44 MAPK is critical for EPAS1 trans-activation.** PC12 cells were exposed to either normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>). *A*, inset panel is a representative immunoblot (from *n* = 6) showing phospho-p42/p44 MAPK immunoreactivity following normoxia (*N*, 21% O<sub>2</sub>) or hypoxia (*H*, 1% O<sub>2</sub>, 6 h). PC12 cells were plated in 24-well dishes and transfected with the HRE-Luc reporter gene (250 ng/well) and either the EPAS1 cDNA (25 ng/well) or the empty expression vector, pcDNA3, as indicated. 48-h post-transfection, cells were exposed to normoxia or hypoxia (1% O<sub>2</sub>, 6 h) in the presence or absence of PD98059 (50 μM), as indicated. Lysates were assayed for luciferase activity as described under “Experimental Procedures.” Data are representative of results obtained in four different experiments. *B*, PC12 cells were transfected with the HRE-Luc reporter gene (250 ng/well), a constitutively active MEK1 construct (pFC-MEK1, 25 ng/well), the EPAS1 cDNA (25 ng/well), or the empty expression vector, pcDNA3, as indicated. Representative experiment showing the effect of constitutively active MEK1 on EPAS1 trans-activation of the HRE reporter gene. Data are from one of three experiments. *C*, data are expressed as fold change from normoxia + EPAS1 and show the relative effect of constitutively active MEK1 on EPAS1-stimulated HRE-Luc activity.

normoxia or hypoxia (1% O<sub>2</sub>) for 6 h. As reported by others (8) we found that expression of EPAS1 increased HRE-Luc activity under both normoxic and hypoxic conditions (Fig. 2A). We also found that inhibition of MEK1, by PD98059, completely blocked the effect of hypoxia on both basal and EPAS1-stimulated HRE-Luc activity (Fig. 2A). These results strongly suggest that the MEK1-MAPK signaling pathway is critical for mediating EPAS1 activation of HRE-dependent gene expression.

To test this, we measured the effect of expressing a constitutively active MEK1 (pFC-MEK1) on basal and hypoxia-induced HRE-Luc activity. MEK1 is a dual specificity protein kinase that directly phosphorylates and activates MAPK (24). Fig. 2B shows that expression of pFC-MEK1-enhanced basal HRE-Luc activity during both normoxia and hypoxia. However, when coexpressed with EPAS1, pFC-MEK1 caused a much larger increase in the *trans*-activation of the HRE-Luc (Fig. 2B). The relative increase in HRE-Luc activity in the presence of pFC-MEK1 and EPAS1 was 13-fold higher than cells transfected with EPAS1 and exposed to normoxia (Fig. 2C). In contrast, transfaction with EPAS1 alone, followed by hypoxia,



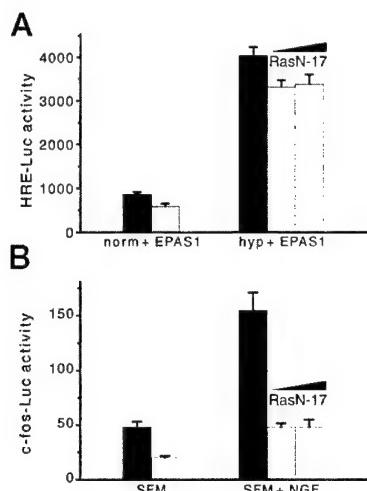
**FIG. 3. EPAS1 protein is phosphorylated and accumulates independently of MAPK.** *A*, PC12 cells were plated in 100-mm dishes and labeled with [<sup>32</sup>P]orthophosphate for 2 h. Cells were then exposed to either normoxia or hypoxia (1% O<sub>2</sub>, 6 h) in the presence or absence of PD98059 (50 μM). EPAS1 was then immunoprecipitated from whole cell lysates and subjected to SDS-PAGE. A representative phosphorimage is shown. *B*, PC12 cells were plated onto 35-mm tissue culture dishes and then exposed to normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>, 6 h) in the presence or absence of PD98059 (50 μM). Following exposure, whole cell lysates were subjected to SDS-PAGE and immunoblotted with an α-EPAS1 antibody. The inset panel is a representative immunoblot showing the effect of hypoxia ± PD98059 on EPAS1 immunoreactivity. Immunoreactivity levels of EPAS1 in the absence (black bars) or presence (shaded bars) of PD98059 are shown. Data are expressed as average percent change from control ± S.E. and represent *n* = 6 dishes performed in two separate experiments.

resulted in only a 2-fold increase in HRE-Luc activity (Fig. 2C).

The increase in EPAS1 immunoreactivity induced by hypoxia was accompanied by a shift in the mobility of the EPAS1 protein (see Fig. 1, inset), suggesting that EPAS1 itself might be phosphorylated during hypoxia. To test this possibility, PC12 cells were pretreated with either PD98059 or vehicle, then metabolically labeled with [<sup>32</sup>P]orthophosphate. Following normoxic or hypoxic exposure, EPAS1 was immunoprecipitated from whole cell lysates, and its phosphorylation state was evaluated by SDS-PAGE and PhosphorImager analysis. Fig. 3A shows that hypoxia does indeed induce phosphorylation of EPAS1. However, EPAS1 phosphorylation was not blocked by PD98059, in contrast to the effects of hypoxia on *trans*-activation of the HRE-Luc reporter gene by EPAS1. We also tested whether MAPK was involved in the induction of EPAS1 immunoreactivity by hypoxia. In these experiments, PC12 cells were pretreated with PD98059 or vehicle prior to exposure to hypoxia. Whole cell lysates were then immunoblotted for EPAS1. Fig. 3B shows that, while hypoxia induced a 13-fold increase in EPAS1 protein levels, inhibition of MEK1 with PD98059 had no effect on the hypoxia-induced accumulation of EPAS1.

Ras is an upstream activator of MAPK (24, 31). In order to test whether Ras was involved in the EPAS1 *trans*-activation of the HRE-Luc, PC12 cells were cotransfected with the EPAS1 expression plasmid, the HRE-Luc plasmid, and increasing amounts of a dominant-negative Ras expression plasmid, RasN-17. Fig. 4A shows that increasing amounts of RasN-17 had no effect on the EPAS1 *trans*-activation of HRE-Luc. However, coexpression of the same amounts of RasN-17 did block activation of a *c-fos-luc* reporter gene by nerve growth factor (NGF) in PC12 cells (Fig. 4B). Thus, EPAS1 activation by hypoxia occurs via a Ras-independent mechanism.

Hypoxia results in depolarization and calcium influx into PC12 cells during hypoxia (17, 32). Consistent with these findings, Egea *et al.* (33, 34) have shown that depolarization of PC12 cells results in MAPK activation via a calmodulin-dependent mechanism. Thus, we hypothesized that calmodulin could be involved in the activation of MAPK and EPAS1 during hypoxia. Fig. 5A shows that pretreatment of PC12 cells with



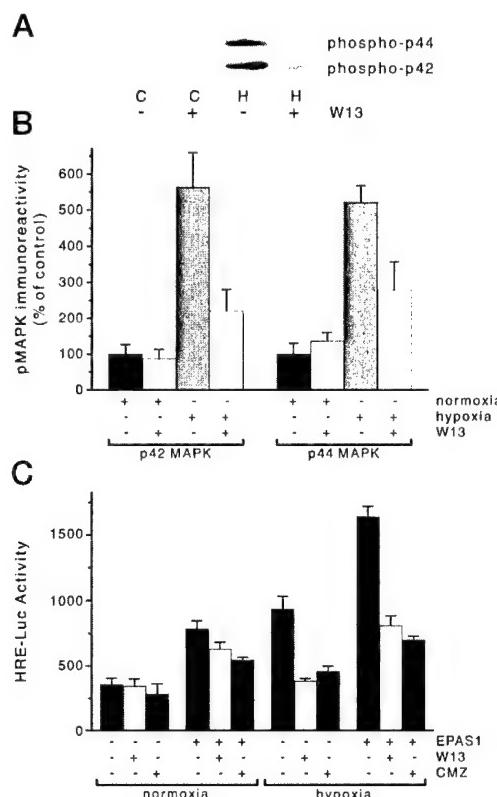
**FIG. 4. Hypoxic activation of the HRE is Ras-independent.** PC12 cells were plated in 24-well dishes and transfected with either the HRE-Luc reporter gene (250 ng/well) or the c-fos-Luc reporter gene (250 ng/well), the EPAS1 cDNA (25 ng/well), and either 50 or 100 ng of the RasN-17, as indicated. pcDNA3 was added where necessary to yield 1  $\mu$ g of DNA/well. *A*, representative experiment showing the effect of dominant-negative Ras on EPAS1 trans-activation of HRE-Luc. 48 h post-transfection, cells were exposed to normoxia or hypoxia and then assayed for luciferase activity, as described under “Experimental Procedures.” Data shown are representative of three different experiments. *B*, representative experiment showing the effect of dominant-negative Ras on c-fos-Luc activity following NGF treatment. 48 h post-transfection, cells were exposed to vehicle or NGF (50 ng/ml) for 6 h and then assayed for luciferase activity, as described under “Experimental Procedures.” Data are from one of three separate experiments.

the calmodulin antagonist, W13 (20  $\mu$ g/ $\mu$ l), caused a pronounced reduction in hypoxia-induced MAPK phosphorylation. These results are shown quantitatively in Fig. 5*B*. We also found that treatment with either W13, or calmidazolium chloride (CMZ, 1  $\mu$ M), another calmodulin antagonist, inhibited both endogenous HRE activity, as well as the EPAS1 trans-activation of the HRE reporter gene (Fig. 5*C*). Thus, MAPK activation of EPAS1 occurs via a calmodulin-dependent pathway, rather than through the proto-typical mediator, Ras.

#### DISCUSSION

Regulation of gene expression by hypoxia is mediated by a number of signal transduction pathways (15, 35). MAPK is known to be critical for the *trans*-activation of many genes and mediates its effects primarily through the phosphorylation of downstream transcription factors (24, 25, 31). The current study shows, for the first time, that EPAS1 is phosphorylated during hypoxia and that the MAPK pathway is critical for EPAS1 *trans*-activation during hypoxia in PC12 cells. While our findings suggest that phosphorylation is an important regulatory step for EPAS1 activation, others have also shown that redox-sensitive changes are critical to the formation of the EPAS1 DNA-binding complex (36). It is likely that EPAS1 activation results from the integration of multiple signals and that the importance of specific signals varies in a cell type-specific manner.

We found that MAPK is required for EPAS1 *trans*-activation of the HRE-Luc reporter gene, as this was completely blocked by PD90859, a selective inhibitor of MEK1 (37), and enhanced by constitutively activated MEK1. However, neither hypoxia-induced phosphorylation nor accumulation of EPAS1 protein was inhibited by PD90859. Thus, although MAPK is critical for hypoxic regulation of EPAS1 function, it is not the kinase that phosphorylates EPAS1 during hypoxia. These results suggest that multiple MAPK-dependent and MAPK-independent signals are required for EPAS1 activation. One MAPK-independent signal leads to accumulation of the EPAS1 protein, presum-



**FIG. 5. MAPK phosphorylation and EPAS1 activity is calmodulin-dependent.** PC12 cells were exposed to normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>, 6 h) in the presence or absence of the calmodulin antagonists W13 (20  $\mu$ g/ $\mu$ l) or calmidazolium (1  $\mu$ M). *A*, representative immunoblot showing the effect of W13 on phospho-MAPK immunoreactivity. *B*, immunoreactivity levels of phospho-MAPK following hypoxic exposure in the absence or presence of W13. Data are expressed as average percent change from control  $\pm$  S.E. and represent  $n = 6$  dishes analyzed in two separate experiments. *C*, representative experiment showing the effect of W13 and CMZ on EPAS1 *trans*-activation of the HRE-Luc gene. PC12 cells were seeded in 24-well dishes and transfected with the HRE-Luc reporter gene (250 ng/well), the EPAS1 cDNA (25 ng/well), or the empty expression vector, pcDNA3, as indicated. Cells were pretreated with W13 (20  $\mu$ g/ $\mu$ l), CMZ (1  $\mu$ M), or vehicle and then exposed to normoxia or hypoxia. Two other experiments gave similar results.

ably by inhibition of ubiquitin-proteasome degradation (38). A second MAPK-independent signal leads to the phosphorylation of EPAS1. Recent evidence by others suggests that multiple signals are involved in regulation of EPAS1 and identifies two domains of EPAS1 that are required for its activation during hypoxia. One of the critical EPAS1 domains is an internal domain that extends from amino acids 450–571 and shares homology with the oxygen-dependent domain of HIF1- $\alpha$  (36, 39, 40). Both the EPAS1 (450–571) and the HIF1- $\alpha$  (oxygen-dependent domain) domains were identified as being critical for the induction of their respective proteins during hypoxia. The second important EPAS1 regulatory domain is a C-terminal activation domain (amino acids 824–876), which is the site of post-translational modification in EPAS1 during hypoxia (36). It is therefore tempting to speculate that phosphorylation of EPAS1 occurs within the C-terminal activation domain of the protein. However, the functional consequences of EPAS1 phosphorylation are unknown and will require further investigation.

The mechanism of MAPK-dependent activation of EPAS1 is unknown. The fact that EPAS1 phosphorylation persists in the presence of PD98059 suggests that the MAPK pathway does not directly target EPAS1, but instead targets other protein(s) that are critical for the formation of the EPAS1 DNA-binding complex. Others have shown that CREB-binding protein (CBP) interacts with HIF1- $\alpha$  and EPAS1 and potentiates the activa-

tion of these proteins (36, 41). Janknecht *et al.* (42) have shown that C-terminal regions of CBP can be phosphorylated by MAPK *in vitro*. Furthermore, Liu *et al.* (43) showed that MAPK can directly regulate the transcriptional activity of CBP following NGF stimulation in PC12 cells. Thus, CBP might be a target of hypoxia-activated MAPK, which could then recruit EPAS1 to the DNA-binding complex. In addition to CBP, the von Hippel Lindau (VHL) tumor suppressor gene product has been shown recently to be involved in the regulation of HIF1- $\alpha$  and EPAS1 protein levels (44). Interestingly, pVHL was also shown to be present in the HIF1- $\alpha$  DNA-binding complex (44). Finally, it has been proposed that several "general transcription factors" are present in the EPAS1 DNA-binding complex (36). These proteins are also potential targets of MAPK regulation. Thus, it is likely that the MAPK-dependent activation of EPAS1 *trans*-activation involves the recruitment of proteins other than EPAS1 to the DNA-binding complex.

The prototypical mechanism of activation of MAPK is via activation of the Ras-Raf-MEK pathway (31). However, some stimuli, such as endothelin-1 and bacterial lipopolysaccharide, have been shown to activate MAPK in a Ras-independent manner (45, 46). Our results indicate that hypoxia is similar to these stimuli, as expression of a dominant-negative Ras had no effect on the ability of EPAS1 to *trans*-activate the HRE-Luc reporter gene.

Since EPAS1 activation was Ras-independent, it was of interest to identify the upstream activators that lead to MAPK and EPAS1 activation. Egea *et al.* (33, 34) have shown that, following depolarization of PC12 cells, MAPK is activated via a calmodulin-sensitive pathway. Exposure of PC12 cells to hypoxia also causes depolarization and calcium influx, via the inhibition of an oxygen-sensitive K<sup>+</sup> channel (17, 32). Our results demonstrate that calmodulin is critical to the activation of MAPK and EPAS1 during hypoxia. Calmodulin is known to activate a number of proteins, including the calcium/calmodulin-dependent family of protein kinases and the calcium/calmodulin-dependent protein phosphatase, calcineurin (47, 48). Future experiments are aimed at defining the mechanism by which calmodulin activates MAPK under conditions of hypoxia. Finally, while the results of Egea *et al.* (33) bear some similarity to our own, their study showed that Ras was critical to the depolarization-induced activation of MAPK and that Ras activation likely resulted from phosphorylation of the epidermal growth factor receptor. In contrast to these results, we have found that MAPK activation by hypoxia was Ras-independent, and we were unable to demonstrate phosphorylation of the epidermal growth factor receptor by hypoxia.<sup>3</sup> These contrasting results illustrate that there may be important differences between hypoxia-induced depolarization and KCl-induced depolarization. In conclusion, our results show that MAPK and calmodulin are critical mediators of hypoxia-induced signal transduction and transcription factor activation. The importance of this calmodulin-dependent pathway is likely to be unique to PC12 cells and other excitable cells, as nonexcitable cells (HEP3B, HEPG2) do not depolarize when exposed to a hypoxic environment.

These results provide the first evidence to define a specific signaling pathway that leads to EPAS1 activation. We show that the MAPK pathway is a critical mediator of EPAS1 activation and that activation of MAPK and EPAS1 occurs through a calmodulin-sensitive pathway, but not through the GTPase, Ras. Further studies are aimed at determining the molecular mechanism by which MAPK regulates EPAS1 function and identifying the endogenous kinase that phosphorylates EPAS1. Such studies will facilitate our understanding of how excitable cells adapt and respond to low oxygen levels. We also show, for

the first time, that EPAS1 is phosphorylated during hypoxia and that this phosphorylation is independent of MAPK.

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<sup>3</sup> T. L. Freeman, unpublished observation.

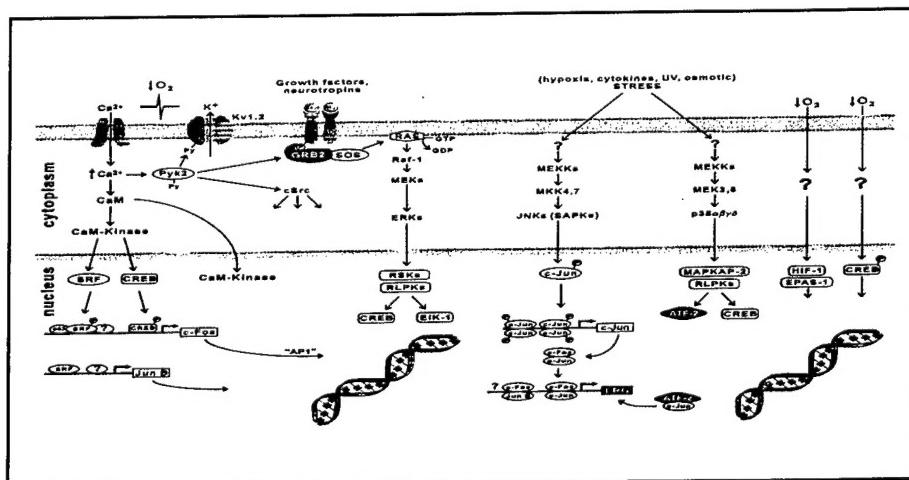
## REGULATION OF SIGNAL TRANSDUCTION AND GENE EXPRESSION BY REDUCED OXYGEN

*In this article David Millhorn discusses recent developments in hypoxia-induced gene regulation with respect to signal transduction pathways and protein-DNA interactions regulating transcription.*

Gene regulation is a complex biological process that results from molecular interactions among nuclear protein factors (transcription factors) and DNA control sequences. These protein-DNA interactions often occur as the result of an extracellular stimulus that is transmitted to the nucleus by a specific signal transduction pathway(s). Although numerous stimuli have been identified which regulate gene expression, perhaps none is more intriguing than reduced oxygen (hypoxia). Hypoxia-induced gene expression has been implicated in a number of physiological processes, including erythropoiesis, carotid body chemoreceptor function, and angiogenesis, all of which enhance the delivery of oxygen to tissue. Genes involved in mediating each of these important processes are normally activated by long-term (hours to days) rather than acute (seconds to minutes) episodes of hypoxia. However, this does not rule-out the possibility that genes can be regulated very quickly by reduced

oxygen. For example, our laboratory measured increased transcription of the *tyrosine hydroxylase* (TH), gene within 15-30 minutes following the onset of hypoxia in pheochromocytoma (PC12) cells (1).

Most of the investigations of hypoxia-induced gene regulation have focussed on either the signal transduction pathways or the protein-DNA interactions that regulate transcription. Here I shall briefly describe recent developments in these areas. A more detailed discussion of hypoxia-induced gene regulation can be found elsewhere (2). An important distinction that should be made when studying signal transduction is whether or not the cells of interest depolarize during hypoxia. Figure 1 summarizes most of the known hypoxia-regulated signal transduction and gene regulatory mechanisms in the excitable oxygen-sensitive PC12 cell line. PC12 cells depolarize during hypoxia due to inhibition of an oxygen-sensitive potassium channel, which we have identified as the Kv1.2 channel (3).



**Figure 1.** Major signal transduction pathways and gene regulatory mechanisms that are regulated by reduced oxygen in PC12 cells.

Depolarization activates voltage-dependent calcium channels leading to translocation of calcium from the extracellular space, which in turn can regulate gene expression via several known calcium-dependent pathways. This is certainly the case in PC12 cells where the hypoxia-induced expression of the immediate-early genes (IEG), *c-fos* and *junB*, and certain late response genes (LRG) such as tyrosine hydroxylase is prevented by removal of calcium from the extracellular milieu or by chelation of intracellular free calcium (4). On-going work in our laboratory suggests that calmodulin-dependent protein kinases (CaM-K) are involved in the calcium-dependent regulation of some hypoxia-responsive genes. In non-excitable cells, translocation of extracellular calcium is lost as a mechanism for gene regulation. However, induced-release of calcium from intracellular storage organelles could be involved.

It is also important to recognize that hypoxia is a metabolic stress which can limit cellular activity. This raises an important question. How do oxygen-sensing cells maintain high levels of activity during chronic hypoxia? The answer to this question is unclear, however it seems likely that oxygen-sensing cells take on a special phenotype that protects them against the harmful effects of hypoxia. Thus, it is entirely possible that *de novo* gene expression mediates hypoxia tolerance as well as specific functions (e.g. erythropoiesis, angiogenesis, chemoreceptor function, etc.) during hypoxia. We recently initiated a series of studies to identify the signal transduction mechanisms and genes that confer the hypoxia tolerant phenotype in PC12 cells. We focussed our studies on the three parallel mitogen/stress-activated protein kinase pathways, which include the mitogen-

activated protein kinase (MAPK), c-jun N-terminal kinase (JNK), and the p38 (*p38 $\alpha$* , *p38 $\beta$* , *p38 $\delta$*  and *p38 $\gamma$* ) kinase pathways. The JNK and p38 kinase pathways have been implicated in the responses to various stressful stimuli such as ultraviolet irradiation and osmotic stress. We found that the p38 and *p38 $\gamma$*  kinase pathways are activated by hypoxia, and that activation of these enzymes is thought to activate nuclear transcription factors. We have also measured an increase in MAPK (*p42/p44*) enzyme activity during hypoxia in PC12 cells, and the involvement of this pathway in regulating transcription of a reporter gene that contains a hypoxia-regulated enhancer (HRE). Thus, signal transduction pathways that have historically been associated with either growth or stress are also regulated by reduced oxygen in PC12 cells. Although we know very little about the genes that are regulated by these pathways or the role of these genes in regulating the cellular response to hypoxia, it is possible that some are involved in conferring a hypoxia tolerant phenotype. Certainly, this is an area that deserves more investigation.

A primary function of signal transduction pathways is to activate protein factors in the nucleus that are involved in regulation of transcription. There has been considerable interest in identifying transcription factors that regulate hypoxia responsive genes. A major breakthrough was the discovery of a transcriptional protein complex called Hypoxia-Inducible Factor-1 (HIF-1), which was first shown to be essential for hypoxia-induced transcription of the erythropoietin (Epo) gene in HEP3B cells (5). HIF-1 is a basic helix-loop-helix (bHLH) PAS domain DNA binding protein that forms a heterodimer with aryl hydrocarbon nuclear receptor

translocator (ARNT). There is growing evidence that HIF-1 is also involved in regulation of genes other than Epo during hypoxia. Another exciting development was the discovery of other bHLH-PAS proteins such as endothelial PAS protein (EPAS-1) that regulate the transcriptional response to hypoxia of certain genes (e.g. VEGF)(6). The signal transduction pathways that regulate the bHLH-PAS transcription factors remain unclear. There is also evidence that other transcription factors (e.g. AP1, CREB, and SRF) might also be involved in regulating gene expression during hypoxia. In addition, we recently discovered a novel kinase pathway that is activated by hypoxia and phosphorylates the cAMP response element binding protein (CREB), a primary transcription factor for a wide variety of genes (7). Thus, activation of transcription might occur via different signal transduction and gene regulatory mechanisms in different tissues and cell types (e.g. excitable vs. non-excitable).

Oxygen is a unique stimulus that readily diffuses throughout the cell. Thus, hypoxia may regulate gene expression by a variety of different mechanisms in different cell types. Major challenges for future research include: 1) identification of oxygen sensory mechanisms, 2) further identification and characterization of oxygen-regulated signal transduction pathways, 3) identification of additional genes that are regulated by hypoxia, and 4) understanding the role that these genes play in regulating the response to hypoxia. Such information will provide new insights into hypoxia-regulated physiological and pathological processes.

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